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Inhibition of glutathione-conjugate secretion from isolated hepatocytes by dipolar bile acids and other organic anions

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The effect of a spectrum of organic compounds on the secretion of a model organic anion, dinitrophenylglutathione (GS-DNP), by hepatocytes was tested. Previous experiments have demonstrated that the secretion of GS-DNP from isolated rat hepatocytes is predominantly mediated by a canalicular transport system for this compound. Preincubation of isolated rat hepatocytes with the bile acids cholic acid (C), taurocholic acid (TC), tauroursodeoxycholic acid (TUDC) and glyco- or tauro-lithocholic acid (GLC or TLC) had no effect on the initial efflux rate of GS-DNP. In contrast, the 3-sulphates of GLC (SGLC) and TLC (STLC) did inhibit GS-DNP efflux; half-maximal inhibition with SGLC was reached with 10 μM. The 3-*O*-glucuronides of both cholate and lithocholate (GlucLC) were even more potent inhibitors of transport; 10 μM-GlucLC inhibited GS-DNP transport by 89%. Other cholephilic organic anions also inhibited GS-DNP secretion, albeit at higher concentrations; at 100 μM, bilirubin ditaurate, an analogue of bilirubin diglucuronide, inhibited transport by 48%. On the other hand, a number of cholephilic cationic and neutral compounds had no effect on GS-DNP efflux. The hepatobiliary secretion of oxidized glutathione (GSSG) was also investigated. In normal isolated perfused rat liver, extensive biliary secretion of GSSG was observed upon intracellular oxidation of reduced glutathione (GSH). GSSG was also actively secreted from isolated normal hepatocytes, and this secretion could be inhibited by 95% by incubation of the cells with 100 μM-SGLC. In contrast, biliary secretion was absent in the isolated perfused liver and in isolated hepatocytes from TR$^-$ mutant rats with a hereditary conjugated hyperbilirubinaemia. These results show that the canalicular efflux of GSSG and GS conjugates can be inhibited by a wide variety of polyvalent organic anions, but not by cations, neutral compounds and unianionic bile acids. This suggests that a multispecific organic-anion transporter is responsible for transport of these polyvalent anions, which is in close agreement with the fact that the biliary transport of all these compounds is defective in the mutant TR$^-$ rat.

INTRODUCTION

A wide variety of organic anions is secreted by the hepatocyte into bile against steep concentration gradients. The uphill transport of such compounds, like bile acids, bilirubin and different glutathione, sulphate and glucuronide conjugates from blood to bile has been extensively documented in the past using experimental systems as bile-duct-cannulated animals or isolated perfused livers (for reviews, see [1,2]. Although canalicular taurocholate transport has partly been characterized at the protein level [3,4], little is known about the protein(s) that are responsible for transport of the other abovementioned anions. Studies with mutant animal models have demonstrated that systems for transport of bile acids and other organic anions are separate entities [5,6]. A more detailed characterization of the transport systems in the canalicular membrane has partly been hampered by the inaccessibility of this specialized membrane domain. We have tried to circumvent this problem by studying organic-anion transport in freshly isolated hepatocytes. In a recent series of experiments [7,9] we have studied the secretion of a model organic anion, 2,4 dinitrophenylglutathione (GS-DNP). On the basis of the following observations it could be concluded that efflux of GS-DNP from freshly isolated hepatocytes is mainly mediated by a canalicular transport system.

(i) In the intact liver the secretion of GS-DNP from the hepatocyte into bile is considerably faster than secretion over the sinusoidal membrane [7], in agreement with previous work by Sies' group (see [8] for a review).

(ii) In the congenitally jaundiced TR$^-$ rat hepatobiliary transport of organic anions, including GS-DNP, is strongly depressed. This transport defect is expressed exclusively at the canalicular side of the cell. For several organic anions (GS-DNP and GSH [7]) and naphthyl glucuronide [10] it was shown that sinusoidal secretion is normal, whereas hepatocanalicular secretion in these animals is absent or extremely low. Efflux of GS-DNP from isolated hepatocytes from the TR$^-$ rat is about 5-fold slower than that from normal hepatocytes. Taken together these results show that at least 80% of the transport activity for GS-DNP from normal hepatocytes represents transport through a system of canalicular origin.

In the present paper we have extended the characterization of this canalicular efflux system in isolated hepatocytes and investigated its substrate specificity. It could be demonstrated that several cholephilic organic anions, including sulphated and

Abbreviations used: GS-DNP, 2,4-dinitrophenyl-*S*-glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; C, cholic acid; TC, taurocholic acid; STC, 3-sulphotaurocholic acid; GlucC, cholic acid 3-*O*-glucuronide; TUDC, tauroursodeoxycholic acid; STUDC, 3-sulphotauroursodeoxycholic acid; TLC, taurolithocholic acid; GLC, glycolithocholic acid; STLC, 3-sulphotaurolithocholic acid; SGLC, 3-sulphoglycolithocholic acid; (Gluc)LC, lithocholic acid (3-*O*-glucuronide); MOAT, multispecific organic-anion transporter; [^{14}C]CDNB, 1-chloro-2,4-dinitro[^{14}C]benzene; LDH, lactate dehydrogenase; t-BOOH, t-butyl hydroperoxide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; TBMA, tributylmethylammonium chloride.

glucuronidated bile acids, inhibit the secretion of the glutathione conjugate, GS-DNP. Furthermore, it could be shown that oxidized glutathione (GSSG) is also transported via this system. These data provide additional evidence for the existence of a multispecific organic-anion transporter (MOAT) in the canalicular plasma membrane of the hepatocyte.

MATERIALS AND METHODS

1-Chloro-2,4-dinitro[^{14}C]benzene ([^{14}C]CDNB; sp. radioactivity; 5.87 mCi/mmol) was obtained from Amersham International (Houten, The Netherlands). Unlabelled CDNB was from Janssen (Beerse, Belgium). Glutathione and glutathione reductase were from Boehringer (Mannheim, Germany 3-Sulpho[^{14}C]glycolithocholic acid (SGLC) and 3-sulphotaurocholic acid (STLC) were prepared as described in [11]. Glucuronidated bile acids were prepared as described in [12]. 3-Sulphotauroursodeoxycholic acid (STUDC) was prepared by a modification of existing methods. Briefly, 7-formylursodeoxycholic acid (2.0 g; obtained according to [13]) was sulphated with SO_3–triethylamine complex in dimethylformamide and conjugated *in situ* with taurine by the EEDQ method, as described in [14]. The crude product was deprotected by treatment with 0.2 M-NaOH in methanol (3.5 equiv.) for 16 h at 25 °C and precipitated by addition of chloroform. The precipitate was re-purified twice by dissolution in methanol and precipitation with chloroform to obtain 0.97 g of the disodium salt, m.p. 193–195 °C [15]. Bilirubin ditaurate was from Porphyrin Products (Logan, UT, U.S.A.). The glutathione conjugate of tetrabromosulphophthalein was prepared and purified as described in [16]. Dibromosulphophthalein was from SERB (Paris, France). Tributylmethylammonium chloride (TBMA) was from Merck (Darmstadt, Germany). All other organic anions, bile acids and t-butyl hydroperoxide (t-BOOH) were from Sigma (St. Louis, MO, U.S.A.).

Animals

Normal male Wistar rats, weighing about 300–350 g, were obtained from Harlan-CPB, Zeist, The Netherlands. Male TR$^-$ mutant rats of the same weight came from our own breeding colonies. This strain has been characterized previously [7,17,18]. The strains described in [5,7,9,11,12,17,18,24] and in [11,12], which were designated TR$^-$ and GY rats respectively, have been isolated and bred independently from the same original Wistar colony (Harlan CPB). As reported, the characteristics of both strains are highly similar. In both strains the mutation was shown to be an autosomal recessive trait. Cross-breeding experiments with both strains gave no complementation and yielded a jaundiced F_1 generation with identical serum bilirubin levels as the F_0 generation (R. P. J. Oude Elferink, unpublished work). Thus the mutation in both strains resides in the same gene (cluster). Probably it involves an identical mutation which occurs at a relatively high frequency in the Wistar colony. All experiments with isolated hepatocytes were carried out with rats that were fasted for 24 h. Isolated perfused livers were from animals fed *ad libitum*.

Isolated hepatocytes

Hepatocytes were isolated by the method of Berry & Friend [19], modified as described in [7]. GS-DNP efflux was measured exactly as described in [9]. Briefly, cells (10 mg dry wt./ml) were preincubated in Krebs bicarbonate buffer [7] in the presence or absence of a compound (as indicated in the Tables and Figures) for 30 min at 37 °C. Subsequently the cells were cooled to 10 °C by incubation for 10 min at this temperature; the cells were loaded with [^{14}C]GS-DNP by incubation with [^{14}C]CDNB (1.5 μmol/g dry wt.) for 10 min at 10 °C. Subsequently the cells were diluted 6-fold with warm medium (37 °C) of the same composition as the preincubation medium. Samples were taken at 30 s intervals from 30 s to 150 s after dilution with warm medium and separated from the medium by immediate centrifugation (10000 *g*) through silicone oil. Radioactivity was determined in the medium fraction (supernatant after centrifugation through oil). Under the conditions described, secretion of GS-DNP was always linear ($r > 0.99$ with linear regression).

For analysis of intracellular metabolites of [^{14}C]CDNB, samples were taken from incubations 30 s after 'warm' dilution of the loaded cells. The cellular fraction was processed and analysed by t.l.c. as described in [7]. Efflux of GSSG from hepatocytes was measured as described in [9]. In all experiments with isolated hepatocytes we measured the release of lactate dehydrogenase (LDH) activity from the cells into the medium and the cellular content of ATP and GSH. In control cells without any addition, we measured an average release of 17 ± 8 % of the total LDH activity during 30 min preincubation (see also [9]).

Isolated liver perfusion was carried out as described in [7]. Livers were perfused with Krebs bicarbonate buffer (without BSA) at a constant pressure of 1.2 kPa (12 cm of water). Bile was collected directly in 10 % $HClO_4$ during 5 min intervals. After a pre-perfusion period of 20 min, t-BuOOH was added to the perfusate at a rate of 3.6 μmol/min during 30 min. Subsequently, the liver was perfused with normal Krebs bicarbonate buffer for 25 min.

Enzymic determinations

For determination of intracellular GSH/GSSG and ATP, the cell fraction (bottom layer in 10 % $HClO_4$ under the silicone oil) was resuspended, centrifuged and the protein-free supernatant of this fraction was neutralized with 3 M-potassium phosphate. ATP was determined fluorimetrically as described in [20]. GSH was measured as described by Tietze *et al.* [21]. GSSG was determined as described by Sacchetta *et al.* [22]. LDH activity was measured as described in [23] in the medium fraction and in the hepatocyte stock solution (for total activity).

RESULTS

Inhibitory effect of organic anions on GS-DNP transport from hepatocytes

It is our hypothesis [7,9] that the postulated canalicular organic-anion carrier is absent or non-functional in the mutant TR$^-$ rat, whereas the transport system for bile acids, such as taurocholate (TC) is present [17]. According to this hypothesis, all compounds of which the transport is defective in the mutant rat are transported by this system and, as a consequence, these compounds should be able to inhibit each other's transport in normal hepatocytes. In order to test this hypothesis we preincubated hepatocytes with different organic anions and measured the effect on efflux of the model compound GS-DNP. Since it was shown that the biliary secretion of some 3-α-sulphated and glucuronidated bile acids is also defective in TR$^-$ rats [12,24], we have measured the effect of such compounds on GS-DNP efflux. Initial efflux rates of GS-DNP from hepatocytes were measured as previously described [9]. Fig. 1 shows a representative experiment in which the efflux of GS-DNP was measured with and without preincubation of the cells with a 100 μM concentration of the 3-sulphate of glycolithocholic acid (SGLC). In both the absence and presence of SGLC, a linear increase in labelled GS-DNP in the medium was observed during the first 2.5 min, indicating that an initial efflux rate is measured.

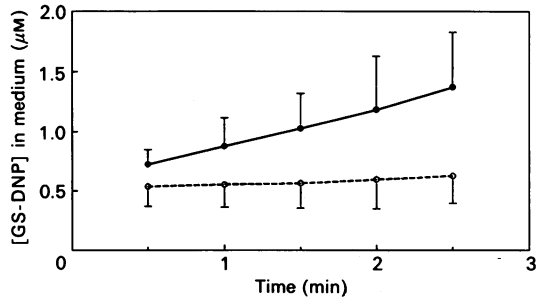

Fig. 1. Inhibition of GS-DNP efflux from normal hepatocytes by SGLC

Normal Wistar hepatocytes were preincubated for 30 min in the presence (○) or absence (●) of SGLC and subsequently loaded with labelled GS-DNP (1.5 μmol/g dry wt.) as described in the Materials and methods section. Efflux was measured upon 'warm' dilution (6-fold) of the cells, followed by rapid separation of cells and medium at the indicated time. Data shown in the Figure are averages ± S.D. from a set of three separate experiments. The average of the calculated efflux rates of GS-DNP in these experiments was 145 ± 38 and 18 ± 10 nmol/min per g dry wt. in the absence and presence of SGLC respectively. Under all conditions efflux was linear during the experimental time interval ($r > 0.99$ with linear regression).

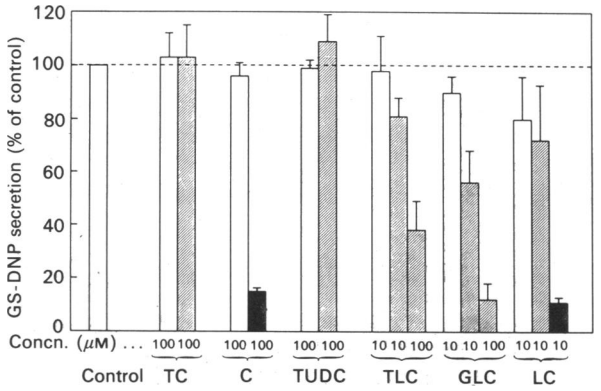

Fig. 3. Inhibition of GS-DNP efflux by bile acids and their 3-sulphates or 3-glucuronides

Hepatocytes were preincubated for 30 min in the presence of the indicated bile acids (□), their 3-sulphated forms (▨) or their 3-glucuronidated forms (■). Subsequently the cells were loaded with GS-DNP and the initial efflux rate from the cells was measured. Values are averages from three experiments and are expressed as percentages (± S.D.) of the efflux rate in the corresponding control incubation without bile acid.

SGLC had a strong inhibitory effect on the efflux of GS-DNP. Fig. 2 shows that SGLC inhibits GS-DNP efflux in a dose-dependent manner, with half-maximal inhibition being reached at 10 μM.

The inhibitory effect of a panel of bile acids and their 3-sulphated and 3-glucuronidated counterparts was tested (Fig. 3). Of this panel, the sulphated forms of the monohydroxy bile acids, lithocholic, glycolithocholic (GLC) and taurolithocholic acid (TLC), had an inhibitory effect. Inhibition of the glycine conjugate was somewhat stronger than that of the taurine conjugate. The unsulphated TLC and GLC could only be tested at the lower concentration (10 μM), because higher concentrations (100 μM) were cytotoxic, as judged by a significant induction of LDH release and a decrease in intracellular ATP concentrations. The unsulphated as well as the sulphated forms of the more hydrophilic bile acids, cholic (C), TC and tauroursodeoxycholic acid (TUDC), had no effect. In contrast with (T)C and 3-sulphotaurocholic acid (STC), the 3-glucuronide of C had a considerable inhibitory effect on GS-DNP efflux (85% inhibition at 100 μM). Also, the glucuronide of lithocholic acid (LC) inhibited transport much more strongly than the corresponding sulphate.

Table 1 shows the effect of preincubation of hepatocytes with different organic anions on the GS-DNP efflux rate. Of the anions, the relatively hydrophobic compounds indocyanine green

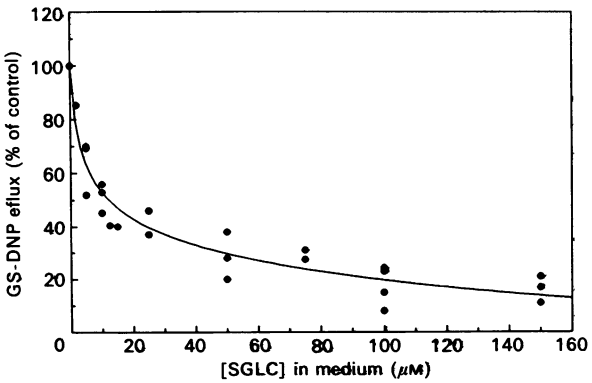

Fig. 2. Dose-dependent inhibition of initial GS-DNP efflux by SGLC

Conditions were exactly as described in Fig. 1. Data were obtained from four independent experiments, and efflux rates are expressed as the percentage of the efflux rate in the absence of SGLC.

Table 1. Influence of different organic compounds on GS-DNP efflux from normal rat hepatocytes

Normal Wistar-rat hepatocytes (10 mg dry wt./ml) were preincubated with the indicated concentration of the inhibitory compounds for 30 min at 37 °C. Subsequently the cells were loaded with GS-DNP (1.5 μmol/g dry wt.) and efflux was measured as described in the Materials and methods section. The data given in the Table are averages ± S.D. from three experiments. The average efflux rate in control incubations was 236 ± 80 nmol/min per g dry wt. Statistical analysis was carried out by a paired Student's t test of the given compound compared with the control incubation: $^*P < 0.05$. Further abbreviations: BDT, bilirubin ditaurate; DBSP, dibromosulphophthalein; ICG, Indocyanine Green.

Preincubation with:	Concentration	Efflux (% of control)
Control		100
Anions		
DBSP	100 μM	96 ± 30
	1 mM	69 ± 10*
GS-BSP	100 μM	91 ± 10
	1 mM	74 ± 12*
ICG	10 μM	94 ± 13
	100 μM	8 ± 5*
Rose Bengal	10 μM	64 ± 3*
Coproporphyrin I	100 μM	68 ± 11*
BDT	100 μM	52 ± 14*
Cations		
Daunomycin	100 μM	101 ± 3
Ajmaline	10 μM	100 ± 4
	100 μM	102 ± 7
TBMA	100 μM	96 ± 4
	1 mM	88 ± 8
Neutral compounds		
Ouabain	1 mM	104 ± 15

(ICG) and Rose Bengal had a very strong inhibitory effect on GS-DNP efflux from the cells while dibromosulphophthalein and the glutathione conjugate of tetrabromosulphophthalein inhibited transport only in the millimolar range. Coproporphyrin I inhibited transport for 30% at a concentration of 100 μM. Daunomycin, a compound that is transported by the multi-drug-resistance system (P-glycoprotein), had no effect on the transport of GS-DNP.

The cations tributylmethylammonium and ajmaline and the uncharged molecule ouabain also had no effect on GS-DNP transport.

In conclusion, these results demonstrate that a wide variety of bivalent organic anions inhibit canalicular GS-conjugate secretion from the hepatocyte.

The efflux assay as used above is based on the fact that hepatocytes can be loaded with labelled GS-DNP by incubation of the cells with [^{14}C]CDNB, which is rapidly conjugated intracellularly with GSH (see the Materials and methods section). It could therefore be that the observed inhibition of GS-DNP efflux is caused by an inhibition of glutathione *S*-transferase, which in fact is very likely in view of the reported inhibition of GS transferase by organic anions [25]. In order to investigate this possibility, we preincubated cells with SGLC and [^{14}C]CDNB as described for Fig. 1. Subsequently the intracellular radioactive metabolites were analysed by t.l.c. These experiments (results not shown) indicate that, under our conditions in both the presence and absence of SGLC, the radioactive CDNB is completely converted into GS-DNP, indicating that the inhibition of efflux of radioactivity is distal to the level of biotransformation. The extent of intracellular conjugation could also be monitored by the change in intracellular GSH content upon incubation of the cells with CDNB. When hepatocytes (10 mg dry wt./ml) were incubated with 50 μM-CDNB, the GSH content fell from 10.2 ± 1.5 to 2.2 ± 0.56 μmol/g. Preincubation of the cells with SGLC or any of the other compounds used in Fig. 3 and Table 1, followed by incubation with 50 μM-CDNB, led to the same decrease in GSH content as was observed in control cells (results not shown). This indicates that, in the presence of all these compounds and under the conditions used, a complete conjugation of CDNB took place. Furthermore, preincubation of the cells with these compounds at the indicated concentrations had no significant effect on the release of LDH into the medium and on the cellular ATP content.

These data strongly suggest that the inhibition of GS-conjugate transport by organic anions is not a secondary phenomenon, but a direct effect on the canalicular transport system.

Canalicular efflux of GSSG in the perfused liver and isolated hepatocytes from normal and TR$^-$ rats

In multiple studies, Sies and co-workers (for a review, see [26]) have demonstrated that GSSG is rapidly secreted into bile and that GSSG and GS-DNP mutually compete for biliary transport, suggesting that these compounds are transported via the same system. We have investigated this hypothesis by measuring canalicular GSSG secretion in both normal and mutant TR$^-$ liver. Livers were perfused with Krebs containing t-BOOH, a compound that oxidizes intracellular GSH. Fig. 4 shows that, in accordance with the results of Akerboom *et al.* [27], infusion of t-BOOH leads to a strong induction of biliary GSSG secretion in normal rat liver. This secretion is completely absent in livers of TR$^-$ rats, indicating that the transport defect in these rats also affects biliary secretion of GSSG.

Subsequently a similar experiment was set up using isolated hepatocytes. Cells from Wistar and TR$^-$ rats were incubated with t-BOOH in order to oxidize intracellular GSH, and the efflux of GSSG was measured. In the absence of t-BOOH, isolated

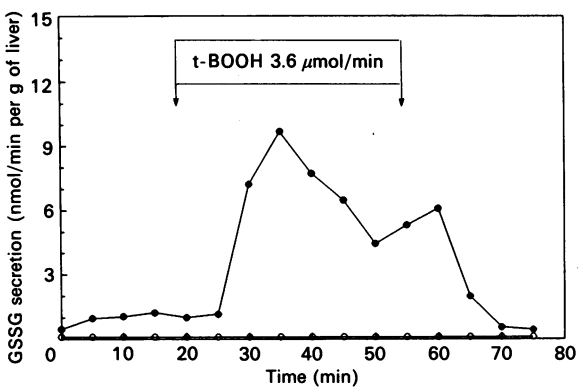

Fig. 4. Biliary GSSG secretion in the isolated perfused liver from normal and TR$^-$ rats

Isolated bile-duct-cannulated livers from a Wistar rat (●) and a TR$^-$ rat (○) were perfused with normal Krebs bicarbonate for 20 min. Subsequently t-BOOH was added to the perfusate to obtain an infusion of 3.6 μmol/min during 30 min. This was followed by perfusion with normal Krebs bicarbonate. GSSG secretion (expressed in GSH equivalents/min per g of liver) was measured as described in the Materials and methods. The data given in the Figure are from a representative experiment from a set of three. Average biliary secretion of GSH equivalents during the t-BOOH infusion period was 5.83 ± 1.77 nmol/min per g of liver in Wistar-rat livers and < 0.05 nmol/min per g of liver in TR$^-$-rat livers.

hepatocytes contained 10.8 ± 1.6 μmol of GSH equiv./g dry wt.; 92% of this was GSH. At 30 s after the addition of 0.5 mM-t-BOOH, 91% of the total cellular content of GSH equivalents was converted into GSSG. In hepatocytes from mutant rats the intracellular GSH content was higher (16.6 μmol/g), in accordance with previous observations [7]. In these cells, however, treatment with t-BOOH led to the same extent of oxidation (90%) as in control cells. In both cell types this state of oxidation lasted for at least 5 min, the time interval during which the efflux of GSSG was measured. During this experimental time interval there was no change in cell viability as measured by release of LDH activity and cellular ATP content. Fig. 5 shows a rep-

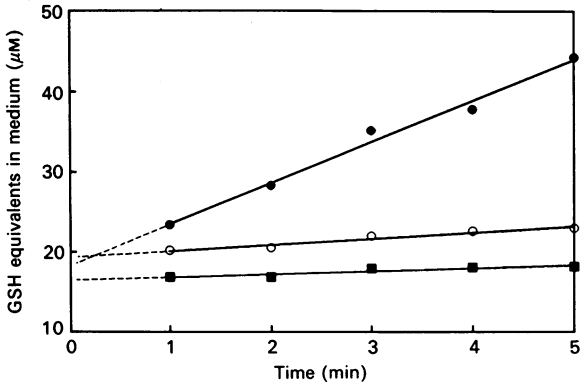

Fig. 5. Inhibition of GSSG efflux from isolated normal hepatocytes by SGLC

Isolated normal hepatocytes (10 mg dry wt./ml) were preincubated for 30 min in the presence (■) or absence (○, ●) of 100 μM-SGLC. Subsequently, either 0.5 mM-t-BOOH (●) or nothing (○) was added, and the efflux of GSH equivalents into the medium was measured during the indicated time period. The data shown in the Figure are from a representative experiment from a set of three; statistical analysis of this set of experiments is given in Table 2. Efflux of GSSG from cells in the presence of t-BOOH was always linear ($r > 0.98$ with linear regression).

Table 2. Efflux of GSH and GSSG from normal- and TR⁻-rat hepatocytes

Hepatocytes (10 mg dry wt/ml) were preincubated for 30 min in the presence or absence of SGLC at 37 °C. Where indicated, 0.5 mM-t-BOOH was added to the suspension and, after 30 s, the efflux of GSH equivalents was measured as indicated in the Materials and methods section. Values are averages ± S.D. from a set of three experiments.

Condition	Rats...	Efflux (nmol/min per g dry wt.) Wistar	TR⁻
Control		124 ± 50	278 ± 71
+t-BOOH		950 ± 335	99 ± 25
+t-BOOH + 100 μM-SGLC		48 ± 28	106 ± 67

resentative experiment carried out with control hepatocytes. In t-BOOH-treated Wistar-rat hepatocytes a rapid linear efflux of GSSG was observed during 5 min (●), whereas non-treated cells secreted GSH equivalents (mainly GSH) only very slowly (○). When the cells were preincubated with 100 μM-SGLC before treatment with t-BOOH, the rapid GSSG efflux was completely inhibited (■). Incubation with SGLC had no effect on the extent of oxidation by t-BOOH. Table 2 shows the compilation of a number of experiments with Wistar-rat and TR⁻-rat hepatocytes. It is clear that the rapid GSSG efflux as observed in Wistar-rat cells in the presence of t-BOOH is completely absent in TR⁻ cells, in both the presence and absence of SGLC. In the absence of t-BOOH, the secretion of GSH equivalents is mainly in the form of GSH. The fact that, under this condition, TR⁻ cells secrete more GSH equivalents than control cells, will be attributable to the fact that the intracellular GSH concentration is higher. Similar to the '*in vivo*' situation, secretion of GSH is predominantly catalysed by the sinusoidal system, which is not affected in the mutant TR⁻ rat. In conclusion, our data show that GSSG is also transported via the transporter that is defective in TR⁻ rats. SGLC inhibits GSSG transport as it does with GS-conjugate transport, which strengthens the hypothesis that all these substrates are transported by the same system.

DISCUSSION

In the present paper we have attempted to characterize the substrate specificity of the hepatocanalicular organic anion-transport system in the rat. The present results suggest that the transport system has a multispecific nature. This is in line with the diversity of substrates that are not, or only poorly, secreted by the mutant TR⁻ rat. However, as long as the exact molecular nature of the defect is unknown, it is possible that not a single carrier, but a family of related carriers with different substrate specificities, is absent or non-functional in the TR⁻ rat, or that a co-factor that is essential for the activity of these transport systems is absent. For this reason we investigated the inhibitory effect of various organic anions on glutathione-conjugate transport in normal rat hepatocytes. Although at very different concentrations, all compounds that are not or very poorly secreted in the TR⁻ rat [11,12,17,18,24] inhibited GS-DNP efflux from hepatocytes of normal rats. These compounds have considerably different molecular structures, but have a few characteristics in common: they all bear at least two negative charges and most molecules have a more-or-less hydrophobic portion. These characteristics are most clear with the bile acids: the bile acids that are not conjugated at the 3-OH group have only one negative charge (at the side chain) and have no inhibitory effect on GS-DNP efflux from hepatocytes. Bile acids that are sulphated or glucuronidated at the 3-OH position bear two negative charges and, of this group, the most hydrophobic species, i.e. LC conjugates, inhibit GS-DNP efflux most. The fact that inhibition by the glucuronides was much stronger than that of the corresponding sulphates may indicate that the glucuronide moiety is a better substrate for transport via the organic-anion carrier. Conjugation of sulphated LC with either taurine or glycine at the end chain did not have much influence on the extent of inhibition.

In accordance with previous studies by others [26,27], our data strongly support the hypothesis that GSSG is transported by the same system as GS-DNP. This is based on two observations: firstly, no canalicular transport of GSSG is detected in the mutant TR⁻ rat, both at the level of the intact liver and at the level of the isolated hepatocyte; secondly, SGLC (100 μM) completely inhibits GSSG secretion from normal hepatocytes. At this concentration the inhibitory effect of SGLC on GSSG transport is stronger than that on GS-DNP transport. This may indicate that the K_m of GS-DNP for the transporter is lower than that of GSSG. Interestingly, the residual secretion rate of GSSG from TR⁻ hepatocytes is very low. In fact, the release of GSSG (in the presence of t-BOOH) from TR⁻ hepatocytes was slower than that of GSH (in the absence of t-BOOH, see Table 2). Since the defect of the TR⁻ rat only affects the canalicular transport of organic anions, it may be concluded that little secretion of GSSG occurs over the sinusoidal membrane of both mutant and normal hepatocytes. Thus, in states of oxidative stress, most GSSG will be secreted into bile.

Several organic anions have been shown to inhibit glutathione *S*-transferase activity [25]. The inhibitory effect of organic anions could therefore also be explained by an inhibition of the transferase activity, since our assay procedure is based on the intracellular formation of GS-DNP by GSH transferase. This possibility was eliminated by demonstrating that the intracellular GSH depletion upon incubation with CDNB was similar in the absence or presence of these organic anions. Furthermore, we have shown that, in the presence of SGLC, the same complete conjugation of CDNB to GS-DNP was obtained. It was previously shown [7] that intracellular conjugation of CDNB in hepatocytes is extremely rapid: addition of 50 μM-CDNB to a hepatocyte suspension (10 mg dry wt./ml) at 37 °C leads to complete conjugation within 30 s. The loading protocol used in the present paper (15 μM-CDNB, 10 mg of hepatocytes/ml at 10 °C for 10 min) will probably give complete conjugation, even in case of extensive inhibition of the transferase activity.

We also tested the inhibitory effect of a number of uncharged and positively charged molecules (Table 1). None of these compounds had an effect on GS-DNP efflux. The absence of inhibition by these compounds cannot be explained by the possibility that they do not enter the cells, since they all have been shown to enter hepatocytes and to be secreted into bile [28–30]. Very recently, West [31] suggested that glutathione conjugates might be transported by the multidrug resistance system (P-glycoprotein). Indeed, this protein (product of the *mdr-1* gene) is present in the canalicular membrane of the human hepatocyte [32], and transport of daunomycin, which is a substrate for this transport system, has been demonstrated in rat canalicular membrane vesicles [33]. Our experiments, however, do not support this suggestion, since daunomycin did not inhibit GS-DNP transport. On the other hand, it is very possible that glutathione conjugates and other organic anions are transported by a 'P-glycoprotein-like' carrier. The sparse data on the characteristics of the canalicular organic-anion carrier suggest a functional similarity between these two systems: both are ATP-dependent systems [9,31] and both transport non-electrolytes. The MDR system is a multispecific transporter for

neutral and cationic compounds. On the other hand, our data suggest that the glutathione-conjugate-transport system is a multispecific system for a wide variety of organic anions.

The extent of inhibition by different organic anions differed strongly: SGLC displayed half-maximal inhibition with 10 μM, whereas with dibromosulphophthalein and bromosulphophthalein-glutathione about 1 mM was needed. There are several possible explanations for these differences. Firstly, transport of bile acids and other cholephilic anions into the hepatocyte leads to an accumulation of these compounds in the cell. The extent of this accumulation in the hepatocyte may differ due to possibly different driving forces. Measurements with ^{14}C-labelled SGLC (results not shown) demonstrated a 20-fold accumulation of this bile acid within the hepatocyte at an initial extracellular concentration of 10 μM. Secondly, most of the compounds used bind to intracellular ligandin (glutathione *S*-transferase) or bile-acid-binding proteins. A different affinity of organic anions for these proteins influences the free cytosolic concentration and thereby the extent of inhibition of GS-DNP transport. Lastly, the various organic anions may have a different affinity for the canalicular transport system. In the experimental system of intact hepatocytes one cannot determine the exact intracellular free concentration of the inhibitory compound. Therefore it is not possible to define the mechanism of inhibition; however, a competitive type of inhibition at the level of the carrier is the most likely explanation. Transport experiments with isolated plasma-membrane vesicles are needed to elucidate this problem. On the other hand, the present study with intact cells gives the best estimation of the inhibitory effect of various organic anions that will be exerted in the '*in vivo*' situation. Indeed, our results indicate that a substantial inhibition of the hepatobiliary transport via the multispecific organic-anion transporter (MOAT) may occur in states of (neonatal) cholestasis where the serum concentration of sulphated LC may be as high as 4.5 μM [34,35]. These are concentrations in peripheral serum, and one may assume that the concentration in portal blood is considerably higher. Similarly, in patients with benign recurrent intermittent cholestasis, an increase in serum sulphated bile acids may play a role in the development of jaundice. Thus secretion of bilirubin, which is one of the physiological substrates for the MOAT, may be impaired by the accumulation of sulphated and glucuronidated bile acids. Furthermore, we have shown that dipolar bile acids also strongly inhibit biliary secretion of GSH in the intact rat [36]. Since GSH in bile probably contributes to the generation of bile-acid-independent bile flow [37], it may be suggested that inhibition of GSH secretion by dipolar bile acids in the above-mentioned disease states may contribute to the development of cholestasis.

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